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Tamaoki's Antigen Factor S_1 ; Its Distribution among Cholera Vibrio Strains and Significance of Its Existence

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Abstract

The antigen factor S_1 which is of a K-antigen nature has been distributed, regardless either of the known O-antigens or of the rough variation by conventional criteria, among classical cholera vibrios, but not among El Tor vibrios. For preparation of accurate diagnostic sera for Inaba and Ogawa serotypes, care should be taken of the existence of this accessory factor. This factor was demonstrable in a soluble state by complement fixation, and the antibody against it took part in the vibriocidal activity. The strain sensitive to complement, irrespective of degree, was found only among the strains carrying this antigen. In the light of this point, the S_1-positive strain seems regarded as "semirough" mutant appeared in the course of long-term storage. This strain was less virulent to the mouse than the S_1-lacking one, but there was no difference among the strains in the vaccine effectiveness whether they possess S_1 or not.

The analytical study on cholera vibrio antigen factor of Tamaoki and his co-workers was first reported in name of Tamaoki, Morii and Yokotani[9] in 1965, then reported in succession down to the nineth report[10] in 1968 and has still been continued. Though there were some diversities as to the details of the method of experiment and consideration of the results obtained for the period above, the principal part of their work remains unchanged and as of the last publication it has been outlined as follows : (1) There is a thermostable K antigen (designated as S antigen) in *Vibrio cholerae*, besides the known O antigens A and B and the thermolabile L antigen common to all cholera vibrios ; (2) The S antigen is subdivided into three factors S_1, S_2, and S_3 according to their distribution among strains ; (3) S_1 among them is found only in the classical cholera vibrio (CV) in disregard of the serotypes Inaba or Ogawa ; (4) S_2 is distributed

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only among Inaba type strains, CV or El Tor vibrio (ET); and (5) S_3 is common to Inaba and Ogawa types, CV or ET.

Since comparatively a small number of reports has been issued concerning the K-properties of cholera antigen and as the possibility of differentiation of CV from ET with S_1 antigen is of the particular interest, the author attempted to verify for himself the results obtained by Tamaoki and his co-workers laying stress on S_1. In the present study, the experiments were also extended to the demonstration of this antigen by means of complement fixation using cell-free antigens and by the vibriocidal test with the factor serum, to the comparison of sensitivity to complement of strains possessing S_1 with that of strains lacking in S_1, and lastly — using representative strains of the limited number of these two antigenic groups — to the comparison of mouse virulence and the vaccine effectiveness of them.

Material and Methods

Strains : Vibrio strains used at the outset of the present study were 32 stock cultures consisting of 20 strains of CV (6 Inaba, 12 Ogawa and 2 Hikojima), of 8 strains of ET (each three Inaba and Ogawa and 2 Hikojima), and of 4 non-agglutinating vibrios (NAG) as controls. As for the strains 761 (CV-Inaba), 35A3 (ditto), 558 (CV-Ogawa), and Maya 3 (ditto) among them which are of importance in carrying out the antigen analysis according to Tamaoki and others, strain Mikage-17 (ET-Ogawa) as well, each two subcultures of the same designation but of different supplier were added. From among the 42 strains in total, 19 strains were selected, giving attention to the exclusion of rough or rugose variants by morphological and physical means and to the confirmation of accuracy of their biological and serological properties related to the type classification. Thus the number of useful strains increased to 94 in the next experiment which dealt with the distribution of S_1 antigen in all the stock vibrio cultures of the author's department.

Antiserum S_1 ; The factor serum B can be prepared constantly by absorbing Ogawa type serum (immunized with living or heated cells) with Inaba type cells (living or heated). But, there remained an agglutinin S_1 other than B which was agglutinable with Inaba type strain 761, when — for example — Ogawa type serum prepared with strain 558 possessing S_1 was absorbed with Inaba type culture 35A3 lacking in S_1. The peculiar agglutination due to S_1 could be observed even if S_1-lacking Ogawa type strain was used for absorption. In such a case the factor B was absorbed already, so that the absorbed serum was available as a monovalent serum for demonstration of S_1 irrespective of the serotype of test-strain.

Antisera S_2 and S_3 : S_2 factor serum is to be prepared by the same method as the known C, the specific antigen of Inaba type, but it has been treated nothing but as one of S antigens on the basis of Tamaoki's view that it is of a K-antigen nature. Another S antigen named S_3 was reported first in the nineth report by Tamaoki et al., 1968.[10] It has been described in this report that there remained an agglutinin reacting weakly with OS-antigens (heated at 100°C for 1 hr) of strains 761, 35A3 and Maya 3 but not reacting with O-antigen of them (120°C for 1 hr), when antiserum "Maya 3-OS" was

absorbed with O-antigen of the same culture and submitted to agglutination. In the present author's study, a S_3 factor serum donated by Tamaoki was used, which had been prepared by immunization with OS-antigen of a B-lacking variant Mikage 17 (originally ET-Ogawa) followed by absorption with O-antigen of strain ET-92 (ET-Ogawa).

Titration of S_1 antibody by complement fixation : This experiment was performed for the purpose of determining solubility of S_1 antigen using four crude whole-cell lysates and three S_1 factor sera. The antigens were prepared by twelve-times repeated freezing and thawing of each two strains carrying S_1, and ten-fold dilutions of them were used for the reaction after being confirmed free from anticomplementary action. The complement fixation was performed by the modification of Kolmer's technique.

Titration of vibriocidal antibody : The method was essentially the same as that of Finkelstein.[2] Twenty sera consisting mainly of sera from rabbits immunized with whole-cell antigens and of the absorbed sera containing various antibody factors were used for the experiment, after being inactivated at 56°C for 30 min. Passive cells to be tested were three strains of Inaba type and four strains of Ogawa type cholera vibrio carrying S_1 or not, respectively. Titration of agglutinating antibody was also carried out in all the cases.

Virulence test : The test was performed twice using dd mice weighing 18 to 23 g, separated into six or nine groups of from four or ten. In the first experiment, each two 24-hr agar cultures (S_1 positive or negative) of Inaba and Ogawa types were tested. The second experiment was that which was corresponding to the unvaccinated control stated in the following experiment on vaccine effectivness. In this case each one strain possessing and not possessing S_1 were inoculated onto brain heart infusion agar slants, incubated at 37°C for 24 hr, and then harvested in saline. Mice were injected into the peritoneal cavity with 0.5-ml volumes of the organism suspensions diluted progressively in two-fold steps, observed for 36 or 48 hr, and the results were expressed with the minimum number of viable organisms that just kill all of several test-animals in a group or with the LD50 culculated by the method of Reed ond Muench.

Protective activity of vaccine : Beef-extract agar, inoculated with strain 558 (CV-Ogawa, S_1 positive) or strain 41 (ditto, S_1 negative), was inoculated at 37°C for 24 hr. The organisms were suspended in saline, the dilutions were made with saline to obtain a final concentration of 5.0×10^8 organisms per ml, and then the organisms were killed by exposure, in Koch steamer, to the temperature of 100°C for 2 hr. Groups of 10 mice were inoculated intraperitoneally with 0.5 ml of the monovalent vaccine above. Eight days later, the animals were challenged with 0.5 ml of the living organism suspensions (diluted progressively in three-fold steps) of the culture used for preparation of the vaccine and with those of the other culture. The results expressed in terms of the LD50 and the results of the unvaccinated control groups calculated by the same method were compared each other, and a discussion was given of significance of S_1 as one of the antigen factors of the vaccine strain.

Results

Occurrence of S-series antigens: There were 17 strains showing spontaneous agglutination in normal saline (especially when the suspension was boiled) among 32 stock cultures and among 10 additional subcultures of the same designation (see "Strains" of Material and Methods), though they were identified all as CV, ET, or NAG by biological means (hemolysis in tubes, Voges-Proskauer reaction, chicken red cell agglutination, trypsin test, colistin test, etc.). Kinds, serotypes, original designations, sources, and suppliers of 19 strains thus selected, and the results of slide and plastic tray agglutination using C, B, S_1, S_2, and S_3 factor sera, inclusive of preparations at the market and those donated by Tamaoki, are summarized in Tables 1 and 2, respectively.

S_1 antigen was demonstrated definitely and unitedly in five strains of CV (761, 558, Kamata, B-1, and Maya 3) regardless of their serotypes Inaba or Ogawa, being ignored a doubtful agglutination in other two strains (VC-13 and B-2) caused only by the factor serum S_1 donated by Tamaoki. The occurrence of S_2 was coincident with that of the factor C characteristic of Inaba type cholera vibrio, but S_3 was observed independently either of the type-specific factors C and B or the other S factors so far as the agglutination was performed on slides. The micro-agglutination on plastic trays of living organisms was practiced in addition, using ten-fold dilutions of Toshiba's factor sera C and B, and of the three S sera donated by Tomaoki. It is worthy of mention that,

Table 1. Strain of vibrios selected and used

Kind	Serotype	Original designation	Source (Supplier)
CV	Inaba	WLL	Japan, 1963, from imported case (NIH, Japan, 1968)
		761*	Bangkok 1957 (Tokuyama QS, 1966)
		35A3*.	NIH, U.S., reference culture (Tokyo QS, 1968)
		VC-13	ditto
CV	Ogawa	558*	ditto (Tokuyama QS, 1966)
		VC-12	ditto (Tokyo QS, 1968)
		Kamata	Japan, 1946 (Kyushu Dental College, 1961)
		A-1	Bangkok, 1958 (Tokyo QS, 1961)
		B-1	ditto
		B-2	ditto
		Maya 3*	ditto (Tokyo QS, 1968)
		VC-1192	406 Lab. (Tokyo QS, 1968)
		VC-41*	NIH, U.S., reference culture (Tokyo QS, 1968)
		VC-90	India, 1961 (NIH, Japan, 1966)
ET	Inaba	PE-1386	Philippines, 1961 (NIH, Japan, 1961)
ET	Ogawa	Mikage-17	Japan, 1962 (Tokuyama QS, 1968)
		SE-1	Sarawak, 1961 (Kyudai, 1964)
NAG		4716	Type 4, Gardner and Venkatraman (Kyudai, 1965)
		8042	Type 2, ditto

* = selected from each among three strains of the same designation but of different supplier.
QS = Quarantine Station.

Table 2. Confirmation of serotype and demonstration of S_1, S_2, and S_3

Strain	Factor serum(slide agglutination) C	B	S_1 (1)	S_1 (2)	S_1 (3)	S_2 (1)	S_2 (2)	S_3 (1)	S_3 (2)	Plastic tray aggl. S(1)
WLL	+	−	−	−	−	+	+	+	+	+(+)
761	+	−	+	+	+	+	+	+	+	+(+)
35A3	+	−	−	−	−	+	+	+	+	+(+)
VC-13	+	−	±	−	−	+	+	+	+	+(+)
558	−	+	+	+	+	−	−	−	−	−(+)
VC-12	−	+	−	−	−	−	−	−	−	−(+)
Kamata	−	+	+	+	+	−	−	−	−	−(+)
A-1	−	+	−	−	−	−	−	+	+	+(+)
B-1	−	+	+	+	+	−	−	−	−	−(+)
B-2	−	+	±	−	−	−	−	+	+	+(+)
Maya 3	−	+	+	+	+	−	−	−	±	−(+)
VC-1192	−	+	−	−	−	−	−	+	+	+(+)
VC-41	−	+	−	−	−	−	−	−	−	−(+)
VC-90	−	+	−	−	−	−	−	+	+	+(+)
PE-1386	+	−	−	−	−	+	+	+	+	+(+)
Mikage-17	−	+	−	−	−	−	−	+	+	+(+)
SE-1	−	+	−	−	−	−	−	+	+	+(+)
4716	−	−	−	−	−	−	−	−	−	−(−)
8042	−	−	−	−	−	−	−	−	−	−(−)

1. Determined by the slide agglutination test. incubated for 15 min at room temperatnre (sign ± denotes doubtful agglutination). The agglutination test using plastic trays was performed in addition by incubating at 37°C for 1 hr (the signs outside parentheses) and in a refrigerator overnight (in parentheses).
2. Factor sera C and B : Diagnostic sera manufactured by "Toshiba" and those prepared by the present author.
3. Sera of S series with an additional remark (1) were given by Tamaoki, with following notes :
   S_1···558(ABS_1)−Mikage-17(ABS_3)=S_1
   S_2···35A3(AS_2S_3L)−Mikage-17 variant (AS_3L)=S_2
   S_3···Mikage-17 variant(AS_3L)−Ogawa-92(ABL)=S_3

only in the case of S_3, there was a marked difference in the results of agglutination tests with regard to the incubation period and the temperature; as shown in the right edge of Table 2, all the cholera vibrios became agglutinable against the factor serum S_3 after putting them in a refrigerator overnight. It can be said in fine that the distribution of S_3 corresponds entirely to that of the group-specific A-antigen.

About one year later of the initial experiments mentioned above, the study on the occurrence and distribution of S_1 was made anew using 94 vibrio cultures being maintained in this laboratory. The agglutination of S_1 was carried out this time quantitatively on plastic trays using bacterial suspensions heated 100°C for 1 hr and serial dilutions of the factor serum S_1 with titer 1 : 320 which was prepared by absorbing 558-OS serum with 41-O cells. The positive reaction of S_1 was given when a test-strain represented the agglutination titer 1 : 80 or more, showing at the same time no spontaneous agglutination in the medium without serum. Number of strains tested and of S_1 positive strains, tabu-

Table 3. Occurrence and distribution of antigen factor S_1

Categories	CV			ET			NAG
	Inaba	Hikojima	Ogawa	Inaba	Hikojima	Ogawa	
No. of strains examined	17	2	34	10	2	17	12
S_1 positive	11	2	21	0	0	0	0
Total	34/53 (64%)			0/29			0/12

lated to their categories, are shown in Table 3. As stated by Tamaoki and as experienced by the present author once, the antigen factor S_1 was found only in CV but not in ET and NAG, though its occurrence was not in all the CV strains tested but in 64 percent.

Experiences in preparing factor sera: In general, the factor serum C has difficulty in preparing as compared with B. The agglutinin C of Inaba type serum was removed little by little by repeating the absorption with Ogawa type culture or it was entirely removed by a single usage of a large quantitity of it. In the case where too small a quantity of the bacterial cells was used, on the other hand, it was very common that there remained something unexplainable agglutinin mixed in agglutinin C. For preparing the factor serum S_1, the following combinations of immune serum and strain for absorption were available: Ogawa-Original/VC-41, Maya 3/VC-41, 558/VC-41, Maya 3/35A3, etc. These absorbed sera yielded the same results in demonstrating S_1 antigen of the test-strains, among which the results of the first two are shown in Table 2. When Ogawa type serum containing agglutinin S_1 and Inaba type strain lacking in S_1 were used, for example the combination of Maya 3 and 35A3 above, an additional absorption with a S_1-negative Ogawa type strain was rendered necessary, and it was possible to prepare the expected factor serum. But the reverse, the absorption of a Inaba type serum with a Ogawa type strain, was not always applicable. The difficulty in preparing the factor serum S_1 in this way is surely connected with the mentioned peculiarity of the agglutinin factor C and/or of the antigen factor C.

Investigation on K-nature of S-series antigens: The next experiment was made,

Table 4. Test on O-inagglutinability and depression of agglutinability by over-heating

Treatment	Suspension medium	Agglutination by the homologous serum of 761	35A3	558	VC-12
uutreated (living)	saline	50-(200)	400 (400)	1600(1600)	1600(3200)
	PBS	200 (400)	400 (400)	1600(3200)	3200(3200)
100°C for 1 hr	saline	800 (800)	800 (800)	1600(1600)	3200(3200)
	PBS	200 (200)	800(1600)	400 (400)	3200(3200)
100°C for 2 hr	saline	50 (400)	800 (800)	800 (800)	3200(3200)
	PBS	50 (200)	800 (800)	400 (400)	3200(3200)
120°C for 1 hr	saline	sp. aggl.	800 (800)	800 (800)	3200(6400)
	PBS	50-(200)	400 (400)	200 (200)	3200(6400)

Figures indicate agglutination titers after incubating at 37°C for 1 hr (outside parentheses), and those after putting it further in a refrigerator for 4 hr (in parentheses).

keeping general conceptions on K-antigen in mind, to ascertain whether the three S antigens are of a K-nature or not, that is to ascertain firstly whether a bacterial culture possessing K-antigen is agglutinative to a very slight degree or not agglutinative in the living state with the homologous O-serum (the O-inagglutinability), and the secondly whether the proper agglutinability disappears or becomes weaker by heating at 120°C (the relatively weak thermostability), while it usually appears when the culture is heated at 100°C for 1-2 hr. Table 4 presents the results concerned with this question. By the quantitative agglutination tests on plastic trays using antigens of various states (living, heated at 100°C for 1 or 2 hr, and at 120°C for 1 hr) of strains 761, 35A3, 558, and VC-12, and the respective homologous O-serum, the O-inagglutinability was observed only in the case where the untreated antigen suspended in saline of strain 761 possessing S_1 was tested with its homologous O-serum. The marked depression in agglutination titer was also observed with this strain, with the other S_1-positive strain 558 as well, when the suspensions were heated at 100°C for 2 hr or at 120°C for 1 hr. The reason why strain 558 did not show O-inagglutinability remains unexplainable, but it must be taken into account that the antigen of these S_1-positive strains differs in some respects from that of those S_1-positive strains because the antigen of the former strains is unstable on exposure to the heat at 120°C.

Using antigens of various states prepared from the two strains possessing S_1, 761 and 558, and ten-hold dilutions of the factor sera C, B, S_1, S_2, and S_3, further agglutination tests were performed on plastic trays (Table 5). In these tests too, the reduction in agglutinability due to overheating was observed so far as the factor serum S_1 was used. It can be said after all that the S_1 antigen, that of Inaba type 761 at least, is of a K-nature.

Solubility of S_1 antigen : To investigate the possibility of demonstration of an antigenic substance showing the same range of occurrence with agglutinogen S_1 in the centrifugal supernatant of cholera vibrio lysate, each of the freezing-thawing lysates of strains 35A3 (Tokuyama QS and Tokyo QS), 558 (Tokuyama), and 41 (Tokyo) were tested reciprocally by complement-fixation with three absorbed sera monospecific for S_1. The complement-fixation test was carried out twice, and the results are shown in Table 6 together with the results of

Table 5. Heat resistance of antigen factors

Strain	Treatment	Factor serum (slide agglutination) C	B	S_1	S_2	S_3
761	untreated	+(卅)	−(−)	卄(卄)	卅(卅)	卅(卅)
	100° 1 hr	卅(卅)	−(−)	±(+)	卄(卅)	卄(卄)
	120° 1 hr	卅(卅)	−(−)	+(+)	卅(卅)	卄(卄)
	100°+120°	卅(卅)	−(−)	−(−)	卄(卄)	卄(卄)
558	untreated	+(+)	卅(卅)	卄(卄)	+(+)	−(+)
	100° 1 hr	−(+)	卅(卅)	+(+)	−(±)	−(−)
	120° 1 hr	±(−)	卄(卄)	+(+)	−(−)	−(−)
	100°+120°	−(−)	卄(卄)	±(+)	−(−)	−(−)

Signs in parentheses : readings after putting in a refrigerator overnight.

Table 6. Complement fixation tests with factor serum S_1 of cell-free antigens

Factor serum S_1		Freezing-thawing lysate 35A3(Tky) (S_1+)	35A3(Tko) (S_1−)	558(Tky) (S_1+)	41(Tko) (S_1−)
558(LOS)−41(LO)**	(1)	4 4 4 1*	4 1 0 0	4 4 4 4	4 1 0 0
	(2)	4 4 2 0	4 2 0 0	4 4 4 3	4 1 0 0
	aggl.t.	(640)	--	(640)	—
558(OS)−41(O)	(1)	4 1 0 0	0 0 0 0	4 1 0 0	0 0 0 0
	(2)	3 1 0 0	1 0 0 0	4 1 0 0	0 0 0 0
	aggl.t.	(320)	—	(320)	—
761(LOS)—35A3(LO)	(1)	4 4 4 1	4 0 0 0	4 4 4 4	4 1 0 0
	(2)	4 4 3 1	4 3 1 0	4 4 4 2	4 1 0 0
	aggl.t.	(1280)	(40)	(640)	—

Tky=Tokuyama QS Tko=Tokyo QS
(1) and (2) : Experiments performed twice.
* Reaction for each tube, in order of serum dilutions 1:40, 1:80, 1:160, and 1:320.
**Immunized and absorbed with living organisms.

agglutination tests which are expressed in terms of agglutination titers. It was apparent from this table that there was a parallelism between the results of the two tests. But, in cases where the two factor sera 558 (LOS)-41(LO) and 761(LOS)-35A3(LO) were used. The complement-fixation resulted as if the sera had contained another kind of antibody reacting commonly on all the lysates used.

Relation of agglutinin S_1 to vibriocidal antibody : The agglutinability and the sensitivity to killing by antibody plus complement of six living cultures (four S_1 carrying and two S_1 lacking), tabulated according to the antibody factor(s) of four non-absorbed and 12 absorbed sera (two of them contain no antibody), are shown un Table 7. Among the factor sera used, the absorbed sera 35A3-761(LOS), WLL-41(O), and 761-35A3(LOS) were not always proper in not bringing about the expected results of agglutination, but there can be no harm in using them as materials for this experiment on account of their minor faults. In case of whole-cell immune sera, the agglutination occurred in high titers, from 1:1280 to 1:10240, irrespective of the serotype and of the agglutination responsible to factors C, B, and S_1 was observed in moderate titers, 1:320-1:1280, as was expected.

The tests for vibriocidal activity of these sera coincided on the whole with the results obtained by the agglutination tests. But, as shown in the results of factor sera 35A3-761(LOS) and 761-35A3(LOS), there can be seen some inferiority of specificity in the results of the vibriocidal tests as compared with those of the agglutination tests. As an unlocked-for consideration, a special attention is to be paid to the sensitivity to complement of strains 761 (Tokyo QS, 1961) and Maya 3 (the same donor, but received in the year 1968) both of which carry the antigen S_1 ; both the strains were sensitive to killing by complement diluted 1:80 (final) without any joint efforts by specific antibodies.

S_1 antigen and sensitivity to complement : The experiment on the sensitivity to complement mentioned above was extended to the remainder of Inaba type cholera vibrio strains. The results of ag-

Table 7. Agglutination and vibriocidal tests of whole-cell and factor sera C, B, and S_1

Serum and factor		Inaba type			Ogawa type		
		35A3 (Tko) (S_1-)	35A3 (Tky) (S_1+)	761 (Tky) (S_1+)	41 (Tky) (S_1-)	558 (Tky) (S_1+)	Maya 3 (Tko) (S_1+)
35A3(Tky)	LAC	7(5)	7(5)	6(s)	6(6)	6(6)	6(s)
761(Tko)	$LACS_1$	7(5)	7(5)	7(s)	7(5)	7(6)	7(s)
41(Tko)	LAB	7(5)	6(5)	7(s)	8(6)	8(6)	8(s)
558(Tky)	$LABS_1$	8(7)	7(7)	7(s)	9(8)	9(8)	9(s)
35A3-761(LOS)	–	–(3)	–(2)	1(s)	–(2)	–(3)	–(s)
41-558(LO)	–	–(–)	–(–)	–(s)	–(–)	–(–)	–(s)
WLL-41(O)	C	5(5)	6(5)	5(s)	–(–)	3(3)	–(s)
35A3-558(LOS)	C	5(3)	5(3)	5(s)	–(–)	–(–)	–(s)
35A3-41(LOS)	C	4(–)	4(–)	4(s)	–(–)	–(–)	–(s)
41-35A3(LO)	B	–(–)	–(–)	–(–)	7(6)	7(6)	7(s)
VC12-35A3(OS)	B	–(–)	–(–)	–(–)	6(6)	6(7)	6(s)
41-761(LO)	B	–(–)	–(–)	–(–)	5(4)	4(4)	4(s)
558-761(LOS)	B	–(–)	–(–)	–(–)	4(4)	4(4)	4(s)
761-35A3(LOS)	S_1	1(3)	6(4)	6(s)	–(3)	5(3)	5(s)
558-41(LOS)	S_1	–(–)	5(2)	5(s)	–(–)	5(3)	4(s)
558-41(OS)	S_1	–(–)	4(2)	2(s)	–(–)	4(–)	2(s)

Figures outside parentheses : Log 2 number of agglutination titer(40=1, 80=2, 160=3, etc.)
Figures in parentheses : Log 10 number of vibriocidal antibody titer. (s) : sensitive to complement with the final concentration of 1 : 80.

Table 8. Agglutinability to S_1 factor and sensitivity to complement

Strain and source	Agglutination titer	Sensitivity to C'			
		1 : 40	80	160	320
Inaba, Original, Denken	320	0	0	0	4
Yanagihara, Biken	320	0	0	0	0
35A3, Tokuyama QS, 1968	320	3	4	4	4
218, Kyudai	320	2	2	4	4
761, Tokyo QS, 1961	320	0	0	4	4
761, Tokuyama QS, 1966	160	2	2	4	4
761, Tokuyama QS, 1968	80	0	0	1	4
Nakagawa, Denken	80	2	3	4	4
H218, Kyudai	40+	4	4	4	4
35A3, Tokyo QS, 1968	40–	3	4	4	4
Yanagihara, Denken	40–	4	4	4	4
WLL, Nagasaki QS, 1965	40–	4	4	4	4
WLL, NIH, Japan, 1968	40–	4	4	4	4
95, NIH, Japan, 1968	40–	4	4	4	4
95, Tokyo QS, 1963	40–	4	4	4	4
VC-13 Tokyo QS, 1968	40–	4	4	4	4
194, Tokyo QS, 1958	40–	4	4	4	4

Estimation of number of colonies : 4=approximately equal to number of colonies in controls ; 3=approximately 75% ; 2=50% ; 1=25% ; 0=less than 25%.

Table 9. Virulence of S_1 positive or negative strains for mice

Strain	No. of viable organisms	No. of deaths	Strain	No. of viable organisms	No. of deaths
Inaba 761 (S_1+)	1.5×10^{10}/ml	4	Ogawa 558 (S_1+)	1.0×10^{9}/ml	2
	1 : 2	4		1 : 2	0
	1 : 4	0		1 : 4	0
	1 : 8	2		1 : 8	0
	1 : 16	0		1 : 16	0
Inaba 35A3 (S_1−)	0.9×10^{10}/ml	4	Ogawa 41 (S_1−)	1.0×10^{9}/ml	4
	1 : 2	4		1 : 2	4
	1 : 4	4		1 : 4	3
	1 : 8	4		1 : 8	3
	1 : 16	0		1 : 16	1

A group consists of 4 mice.

glutination tests of 17 strains on the S_1-serum with titer 1:320, prepared by absorption of 558(LOS) serum with strain 41(LO), and the results of the tests for complement sensitivity using four doubling dilutions from 1:40 to 1:320 (final) of the complement are shown in Table 8. In accordance with the agglutination titers, these test-strains were classified into two groups and one strain, namely the first group composed of eight strains which possess certainly S_1 antigen, the second group composed of eight strains lacking in S_1, and one strain, H218-Kyudai, manifesting the S_1-agglutination of very low titer. Including the last strain in the second group, it can be said that all the S_1-negative strains resist to killing by complement. On the other hand, in the S_1-positive group there were three strains highly sensitive to complement, three strains moderately sensitive, and one strain (35A3, Tokuyama, 1968) indicating the same resistance to complement as that shown by one strain (35A3, Tokyo, 1968) of the S_1-negative group. In addition to the existence of this exceptional case, it is not to be disregarded that no correlation could be acknowledged between the degree of agglutination and that of complement-sensitivity in the strains of the S_1-positive group. In any case, it may well be said that the strain sensitive to killing by complement is often found among strains carrying S_1 antigen.

S_1 antigen, virulence, and vaccine effectiveness : In the out-set, comparison was made of virulence to mice between two S_1-positive strains Inaba-761 and Ogawa-558 (Tokuyama QS, 1966), and two S_1-negative strains Inaba-35A3 and Ogawa-41 (Tokyo QS, 1968) (Table 9). The available data for this comparison between the two strains of Ogawa type can also be seen in the column "unvaccinated control" in Table 10. It it apparent from these findings that S_1-negative strains 35A3 and 41 were more virulent to the mouse than strains 761 and 558 possessing S_1, though there was a passable difference between the results of the first and the second experiments which were performed at interval of one year and four months ; the LD50 was hardly calcu-

Table 10. Resistance of mice immunized with S_1 positive or negative strains

Immunized with	Challenged with 558		Challenged with VC-41	
	No. of viable organisms	No. of deaths	No. of viable organisms	No. of deaths
CV-558 (S_1+)	2.1×10^{10}	10*	2.8×10^{10}	10
	1 : 3	9	1 : 3	10
	1 : 9	8	1 : 9	10
	1 : 27	1	1 : 27	10
	1 : 81	0	1 : 81	0
	1 : 243	0	1 : 243	0
	1 : 729	0	1 : 729	0
	1 : 2187	0	1 : 2187	0
	1 : 6561	0	1 : 6561	0
	($LD50 = 1.6 \times 10^9$/ml)		($LD50 = 5.9 \times 10^8$/ml)	
CV-41 (S_1-)	2.1×10^{10}	10	2.8×10^{10}	10
	1 : 3	10	1 : 3	10
	1 : 9	8	1 : 9	10
	1 : 27	1	1 : 27	10
	1 : 81	0	1 : 81	10
	1 : 243	0	1 : 243	1
	1 : 729	0	1 : 729	0
	1 : 2187	0	1 : 2187	0
	1 : 6561	0	1 : 6561	0
	($LD50 = 1.5 \times 10^9$/ml)		($LD50 = 5.3 \times 10^8$/ml)	
unvaccinated control	2.1×10^{10}	10	2.8×10^{10}	10
	1 : 3	10	1 : 2	10
	1 : 9	10	1 : 9	10
	1 : 27	6	1 : 27	9
	1 : 81	1	1 : 81	6
	1 : 243	0	1 : 243	3
	1 : 729	0	1 : 729	0
	1 : 2165	0	1 : 2165	0
	1 : 6561	0	1 : 6561	0
	($LD50 = 6.1 \times 10^8$/ml)		($LD50 = 2.9 \times 10^8$/ml)	

*No. of deaths to 10 mice injected.

lated in the first experiment, but the results obtained from the comparison of viable counts necessary for killing all the mice in a group suggested that the S_1-negative strains had at least four times as much virulence as the S_1-positive strains, and in the second experiment it was shown clearly by the presentation of LD50 that the virulence of strain 41 (S_1-) was twice that of strain 558 (S_1+).

The results obtained from the active immunization with heat-killed vaccines (2.5×10^8 organisms, intraperitoneal, at one time) prepared from strains 558 (S_1+)

and 41 (S_1-) followed by challenge inoculation with the homologous and the heterologous vibrios are shown in the main part of Table 10. When these results were compared with those of the control group, some small protection was observed, in common to four possible combinations of the vaccines and the inocula, but there was no difference noticed in their protective activity between the two vaccines. The difference in virulence between strains 558 and 41 was more conspicuous in these immunized animals than those unimmunized.

Discussion

Among the three S antigens advocated by Tamaoka, the factors S_2 and S_3 were of an O-antigen nature, contrary to his opinion, and that the factor S_2 covered almost all the strains possessing the known type-specific factor C and S_3 quite all the strains of CV and ET. Consequently, the author can hardly approve of Tamaoki's presentation of these two antigen factors with the view of increasing complication in serology of *Vibrio cholerae* and in typing practice. In the present studies, experiments were pushed forward only on the factor S_1, now that the state of affairs was made clear. The following conclusions could be drawn out as to S_1 from the evidence mentioned above :

1) The factor S_1 has been distributed, regardless of the known O-antigens A, B, and C, among the classical cholera vibrios, but not among El Tor vibrios.

2) It was resistant to heating for 1 hr at 100°C but not to autoclaving for 1 hr at 120°C. The O-inagglutinability was observed in part of these strains. Therefore, this antigen was considered to be a K-antigen, rather than an O-antigen.

3) It was detectable in a soluble state by complement-fixation with a monovalent serum containing the agglutinin anti-S_1.

4) Its serological specificity could be observed also by the vibriocidal test, in the same manner as the type-specific factors C and B.

5) The strain sensitive to killing by complement was often found among the strains carrying S_1.

6) The S_1-positive strain was less virulent to the mouse than the S_1-negative one.

7) There was no difference in the vaccine effectiveness between the strains possessing S_1 or not.

The finding that S_1 could be found only among CV strains — though not among all of them, but at the rate of two-thirds —, may throw some light for the serological classification of cholera vibrio, as no antigen factor has been known yet for the distinction between CV and ET. An anxiety is manifested, however, regarding the possibility of the antigenic variation in the course of long-term storage of the strains. One must pay attention first to the fact that most of CV strains used in the present study were those of old isolation while ET strains were all those isolated in the 1960's in the Far East, and secondly that there was the discrepancy in existence of this antigen between two or among three strains of the same designation but received from different donors. As shown in Tabel 8, three strains designated 761 and two strains designated 35A3 were tested for S_1-agglutination and for sensitivity to complement, among which strains 761 showed different titers in the

agglutination and one strain among 35A3 was determinded as S_1-positive but another S_1-negative. It can be said, at least, that the existence or non-existence of this antigen in some definite strains was consistent during the course of the present study.

Although the reasons for the discrepancy in demonstration of the antigen S_1 among the subcultures originated from their parent strain and why one-third of the CV strains does not carry this antigen are not to be explained, it can safely be said that at last the antigen S_1 bears no relationship to rough variation in a popular sense, which is namely the variation demonstrable by morphological observation of colonies and demonstrable by some physical means. As for the negation of the variation of this type by the latter means only the spontaneous agglutination in saline solution and enhancement of it by heating at 100° or 120°C were taken into consideration, and the strains of smooth form screened thus were used throughout the experiments in the present study. The precipitation of the cells derived by adding Millon's reagent or acridine dye, or by placing the cell-suspension in a refrigerator overnight, took place in all the test-strains with or without S_1. This state of affairs may be natural as for long-term stock cultures of cholera vibrio.

It has been pointed out by some investigators (Dudani[1] ; Fukumi, Ohashi and Shimada[3] ; Singh and Ahuja[7]) that rough form of cholera vibrio is sensitive to complement. Viewed from this point together with the author's finding that S_1-positive strains were for the most part sensitive to complement, the strain possessing S_1 may be considered to be a kind of rough mutant (though not fully rough by conventional criteria, but to be called "semirough"), and this point of view has been supported by the fact of relatively less virulence of this type strain (the author) which coincides with the generally accepted view (e. g. A. Mukherji and V. Mukherji[4]).

It is noteworthy that immunization of mice with heat-killed vaccines prepared from the S_1-positive complement-sensitive strain and from the S_1-negative complement-resistant strain elicited nearly the same degree of protection against challenge with the homologous and the heterologous organisms, and among these immunized animals more remarkable differences in virulence was observed between the two kinds of strains than in those unimmunized animals. This is considered to be an important finding which may concern with the immunity mechanism of cholera vibrio, but because of insufficient experimental data, for example due to the failure of measurement of antibodies on experimental animals, the author regrettably can not give any explanation for it. In recent years, discussions are going on among some investigators (Ornellas et al.[5] ; Roantree[6] ; Steward et al.[8]) about the immunological properties of complement-sensitive and complement-resistant *Salmonella* or *Escherichia coli*. Referring to these studies, continued studies on the roll of S_1-antigen as an immunogen are necessary.

Strains 761, 35A3, 558, 41 etc. are well-known stocked cultures of cholera vibrio which have often been used not only for experimental studies in this field, but also practically for preparation of diagnostic sera and prophylactic vaccines. The needs for selection of suitable strains in consideration of S_1 in preparation of diagnostic sera, particularly factor sera, have been already stated by Tamaoki and his co-workers, and

the present author (Horikawa) also agree to their opinion on the basis of the studies presented here. It must be kept in mind that the existence of S_1 in cholera vibrio cultures sometimes hinders the progress of animal experiment or the preparation of correct diagnostic factor sera.

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玉置の抗原因子S_1，そのコレラ菌における分布と存在意義

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摘　　要

抗原因子 S_1 はK抗原としての性状をもち，既知O抗原ともまた通例の形態・理化学判定規準によるR型変異とも関係なく，クラシックコレラ菌株に分布しているがエルトールコレラ菌株には分布していない．稲葉，小川血清型別用の正確な因子血清を調製するに当ってはこの附加因子の存在に顧慮を要する．本因子は補体結合反応で溶性の形で立証でき，また本因子に対する抗体は殺ビブリオ性に関與していた．程度の差こそあれ補体に対し感受性を示す菌株は本抗原を有していた．この点からみると，S_1 陽性菌は菌株の長期保存中に発現したセミR変異菌とみなしえるようである．この菌株は S_1 欵のものよりマウスに対する毒力が低いが，一方，ワクチン効果については免疫原の S_1 の有無によって差異を認めなかった．